Circulating cell-free DNA in hematological malignancies

Lieselot Buedts[1] and Peter Vandenberghe[2]

[1]Center for Human Genetics; and [2]Department of Hematology, University Hospitals KU Leuven, Belgium

E-mail: peter.vandenberghe@uzleuven.be *doi:10.3324/haematol.2015.131128*

In 1948, Mandel and Métais detected nucleic acids in peripheral blood plasma, thereby invalidating the previous dogma claiming that DNA is always cell-bound.[1] However, long before the double helix structure of DNA was discovered, their observation fell into oblivion for many decades. In the nineties, the interest in circulating cell-free DNA (ccfDNA) resurged, as ccfDNA was shown to contain DNA derived from the fetus, or more precisely the placenta, in pregnant women, and from cancer cells in cancer patients.[2,3]

The majority of ccfDNA is thought to originate from apoptosis, as evidenced by the predominant fragment length of (multiple integers of) 160 to 180 bp, typical of DNA released from apoptotic cells. However, the presence of larger fragments, up to 21 kb, suggests that ccfDNA can also be derived from necrotic cells.[4] Moreover, many studies attribute a notable fraction of ccfDNA to an active secretion mechanism, releasing newly synthesized nucleic acids associated with lipid-protein complexes.[5] Since these processes occur ubiquitously in the body, the blood plasma of healthy individuals by default contains ccfDNA at steady-state levels and primarily derived from hematopoietic cells.[6] In cancer patients, ccfDNA also contains, besides ccfDNA derived from non-malignant cells, a fraction of circulating tumor DNA (ctDNA) as tumor cells also shed DNA into the circulation. This creates an opportunity for "remote sampling" of the tumoral DNA, eloquently epitomized as the "liquid biopsy". As such, analysis of ccfDNA may potentially provide insights into somatic genomic mutations of the patient's tumor with potential diagnostic, prognostic and therapeutic value. In addition, the relative amount of ctDNA can be informative on the tumoral burden and its evolution. Generally, ctDNA constitutes only a minor fraction (less than 1%) of the total ccfDNA, although the actual fractions can range from 0.01% to more than 90% depending on the tumor size, location and vascularity, and the disease extent and stage.[7] Given the high amount of background ccfDNA and the low amount of input material, molecular methods with a low detection threshold are required for the detection of rare tumor variants. For metastatic tumors such methods have been validated.[8,9] In the context of early-stage malignancy or minimal residual disease, approaches with lower detection thresholds are required, which are capable of identifying mutations in limited amounts of ctDNA.[8,10,11]

For solid tumors, several studies have validated that somatic copy number variations and somatic mutations detected in ctDNA match with those present in the primary tumor. For instance, *KRAS* mutations have been detected in the ccfDNA of colorectal cancer patients with a concordance of 96% with the mutation status in tumor tissue samples,[12] and *HER2* amplifications have been identified in the ccfDNA of 64% of cases with *HER2*-amplified breast cancers.[13] In this way, ccfDNA can facilitate diagnostic and prognostic cancer assessment by noninvasively retrieving the tumor's somatic mutations. Furthermore, while conventional biopsies comprise only one lesion or part of one lesion, ctDNA DNA is derived from the entire primary lesion as well as from metastatic lesions, as they all shed ctDNA. Thus, the ctDNA is in principle representative of the whole tumor burden within the patient, and less subject to sampling bias. This is of pivotal importance for solid tumors as molecular tumor heterogeneity is known to exist between the primary and metastatic sites as well as within tumoral lesions.[4] A second potential application is the follow-up of disease under therapy: somatic mutations and structural chromosomal alterations first identified in primary tumors, and confirmed in ctDNA, can subsequently be targeted in ctDNA in a quantitative matter, to estimate the evolution of the tumor burden under therapy. In this context, ccfDNA sampling offers a cheaper and less interventional approach in comparison with PET/CT scans; this may enable frequent sampling so that patients can be monitored more closely or dynamically.[14] Another promising application is monitoring the emergence of resistance mutations. Diaz *et al.* spotted resistance *KRAS* mutations in the ctDNA of colorectal cancer patients 5 months before radiological evidence for tumor progression.[15] Finally, the analysis of ccfDNA in the context of noninvasive prenatal testing has led to a low rate of incidental diagnoses of cancer.[16,17] As such, the potential clinical applications of ccfDNA in a context of malignant diseases may, in principle, encompass almost the entire course of cancer from early detection to diagnosis, therapeutic monitoring and monitoring of relapse.

Hematological malignancies generally are "liquid" malignancies, originating in the bone marrow or in the lymphoid system, and circulating in the peripheral blood, in leukemic numbers or in lower numbers detectable only by flow cytometry or molecular techniques. As such it is not surprising that hematologists have, by intuition, taken the straightforward route of examining cellular fractions from blood or bone marrow for cytological, immunophenotypic or genetic studies at diagnosis or during follow-up.[4] Nevertheless, the fact that *BCL-2/IgH* PCR status at the end of induction treatment is not predictive for progression-free survival in relapsed/resistant follicular lymphoma clearly indicates that sampling the cellular fraction of blood has limitations.[18] Indeed, more recent findings indicate that ccfDNA may hold several advantages for hematological disorders that were unanticipated until recently.

In a technical prospective proof-of-principle study, we recently described that low-pass sequencing of ccfDNA from 10 consecutive patients with newly diagnosed Hodgkin lymphoma (HL) revealed copy number changes present in Hodgkin/Reed-Sternberg (HRS) cells in the majority of cases, including early as well as late stage Hodgkin lymphoma. The initial case of the above series of ten came to our attention as an incidental finding revealed by noninvasive prenatal testing. Among the initial 4000 noninvasive prenatal tests (NIPT), we identified three cases with genomic profile aberrations corresponding to tumor-related copy number variations. Further clinical evaluation revealed a maternal ovarian carci-

noma, Hodgkin lymphoma and follicular lymphoma.[16] Given that the finding of Hodgkin lymphoma was especially surprising, we initiated a pilot study in which nine additional patients with nodular sclerosis Hodgkin lymphoma at diagnosis and without concomitant malignancies, were recruited.[19]

Hodgkin/Reed-Sternberg cells, the malignant hallmark cells in classical Hodgkin lymphoma are embedded in a dense cellular infiltrate of B and T cells, macrophages, neutrophils and other cell types, and generally represent only 0.1-2% of the tumor mass. The low abundance of HRS cells in Hodgkin lymphoma tissue represents the major technical obstacle for the exploration of their genome. This led researchers to study HRS-derived cell lines, or HRS cells purified by laser microdissection[20] or by flow-sorting[21] from primary lymph node tissues. Increased concentrations of ccfDNA had been observed in classical Hodgkin lymphoma patients before, although it was not formally proven that this ccfDNA contained DNA from HRS cells, along with ccfDNA from non-malignant cells.[22] Our findings therefore revealed a potential novel gateway for the exploration of the genetic landscape of Hodgkin lymphoma on a wider scale than was previously possible. Exploitation of this gateway on a wider scale in the context of clinical studies may facilitate the recognition and identification of genetic subgroups of HL, and hence the development of personalized therapies. Analysis of ccfDNA may also hold promise for other hematological malignancies where the low malignant cell fraction in disease tissues may challenge their genetic characterization, e.g. in T-cell/histiocyte-rich B-cell lymphoma, or multiple myeloma, or in any situation where primary lesions are difficult to approach for technical reasons.

In our study, copy number aberrations were detected in 9 out of 10 cases, two of whom had stage IVB and seven of whom had stage IIA disease. Interestingly, the aberrations were most pronounced in the two cases with extensive disease. Moreover, it was found that the genomic representation profiles normalized between day 15 and 28 of the first chemotherapy course. Both observations strongly suggest that the fraction of ctDNA in circulating cell-free DNA is related to the malignant disease burden in HL, and this is now the subject of our further investigations. In this respect, the half-life of ctDNA is known to be short, up to less than an hour:[4] this may turn out to be a very attractive feature, as it might allow for the dynamic monitoring of tumor burden in time spans of hours to days instead of weeks to months. This early window by far antedates the decline of protein biomarkers, such as paraproteins or other tumor markers as well as the tumor response as evaluated by conventional and even metabolic imaging. Though the exact clearance mechanism of ccfDNA remains poorly understood, plasma nucleases are probably involved in the degradation of ccfDNA, complemented by lymphatic clearance and transrenal excretion.[23]

That ctDNA analysis holds great promise for the follow-up of the malignant burden in non-Hodgkin lymphoma (NHL), was excellently demonstrated by a retrospective analysis by Roschewski and colleagues of ctDNA in diffuse large B-cell lymphoma (DLBCL), one of the most common types of non-Hodgkin lymphoma. Because of the considerable risk (40%) of relapse and treatment failure in the first five years after diagnosis, patients are usually followed up to 5 years after first-line treatment using PET/CT scans.[24] Indeed, while detecting recurrence at the subclinical stage could be beneficial as tumor burden is still likely to be low, the potential benefits of PET monitoring are counterbalanced by additional health risks for patients due to ionizing radiation exposure, increased medical costs and false positive results.[14] Roschewski and colleagues hypothesized that massive parallel sequencing of *VDJ immunoglobulin* gene sequences in ccfDNA could serve as a more convenient and more sensitive alternative for monitoring the tumor burden.

Mature B cells have uniquely rearranged immunoglobulin gene loci. Roschewski *et al.* identified the tumor-specific *VDJ* rearrangement, the 'clonotype', in pretreatment lymph node biopsies or cffDNA in serum samples, and measured the tumor clonotype in cffDNA during and up to 5 years after first-line treatment to quantify the fraction of ctDNA. Detectable interim circulating tumor DNA had a positive predictive value of 62·5% (95% CI 40·6–81·2) and a negative predictive value of 79·8% (69·6–87·8), respectively. Surveillance monitoring of circulating tumor DNA was carried out in 107 patients who achieved complete remission. This analysis showed that the hazard ratio for clinical disease progression was 228 (95% CI 51–1022) for patients who developed detectable circulating tumor DNA during surveillance compared with patients with undetectable circulating tumor DNA. Surveillance circulating tumor DNA had a positive predictive value of 88·2% (95% CI 63·6–98·5) and a negative predictive value of 97·8% (92·2–99·7), respectively, and identified the risk of recurrence at a median of 3·5 months (range 0–200) before evidence of clinical disease. The authors concluded that interim ctDNA is a promising biomarker for identifying patients at high risk of treatment failure. In addition, surveillance of ctDNA in remission identified patients at risk of recurrence before clinical evidence of disease relapse.[25]

This relatively new area of research still faces important technical challenges. The standardization of ccfDNA analysis is required, as is the elucidation of the unknowns, such as inter-individual and diurnal variation, baseline levels, and the effect of inflammatory responses or physical trauma on ccfDNA levels.[4] It is equally unclear to what extent physical barriers, such as the blood-brain barrier or the blood-testis barrier, impair the release of ctDNA from tumors in these sanctuary sites.[9] Moreover, the low fraction of ctDNA in early stage disease and the poor extraction efficiency require a considerable sequencing sensitivity for oncogenic mutations to be reliably distinguished from sequencing errors.[10] For targeted detection of known mutations, several powerful methods are at hand based on digital PCR, assessing each ccfDNA molecule individually in emulsion droplets or microfluidic devices to reduce the background noise of normal ccfDNA.[26] Finally, for unbiased mutation discovery, the error rate of the current NGS platforms, of around 0.1%, is problematic.[27] Alternatively, several methods have been developed to improve template preparation or data interpretation, either by using barcodes allowing PCR- and sequencing-induced mutations to be filtered out after sequencing (TAm-Seq,[8] SAFE-SeqS,[10] Duplex Sequencing[11]), or by enriching tumor-specific mutations prior to library preparation (COLD-

PCR,[28] Nuclease-Assisted Mutation Enrichment[29]).

The last decades have witnessed significant progress in elucidating the origin, characteristics and potential applications for the analysis of ccfDNA. Given its ability to assess dynamic and comprehensive tumor processes, ccfDNA, as a source for ctDNA, holds great promise for the implementation of personalized and dynamic cancer therapies. In addition, the low detection limit and ease of sampling may critically improve post-treatment monitoring compared to surveillance imaging. Resolving the remaining technical and practical challenges will allow ccfDNA to prove its clinical utility and revise cancer patient care.

References

1. Mandel P, Metais P. Les acides nucléiques du plasma sanguin chez l'homme. C R Seances Soc Biol Fil. 1948;142(3-4):241-243.
2. Vasioukhin V, Anker P, Maurice P, Lyautey J, Lederrey C, Stroun M. Point mutations of the N-ras gene in the blood plasma DNA of patients with myelodysplastic syndrome or acute myelogenous leukaemia. Br J Haematolo. 1994;86(4):774-779.
3. Lo D, Corbetta N, Chamberlain P, et al. Presence of fetal DNA in maternal plasma and serum. Lancet. 1997;350(9076):485-487.
4. Schwarzenbach H, Hoon DSB, Pantel K. Cell-free nucleic acids as biomarkers in cancer patients. Nat Rev Cancer. 2011;11(6):426-437.
5. Bronkhorst AJ, Wentzel JF, Aucamp J, Van Dyk E, Du Plessis L, Pretorius PJ. Characterization of the cell-free DNA released by cultured cancer cells. Biochim Biophys Acta. 2016;1863(1):157-165.
6. Lui Y, Chik K, Chiu R, Ho C, Lam C, Lo Y. Predominant hematopoietic origin of cell-free DNA in plasma and serum after sex-mismatched bone marrow transplantation. Clin Chem. 2002;48(3):421-427.
7. Diehl F, Schmidt K, Choti MA, et al. Circulating mutant DNA to assess tumor dynamics. Nat Med. 2008;14(9):985-990.
8. Forshew T, Murtaza, M. Noninvasive identification and monitoring of cancer mutations by targeted deep sequencing of plasma DNA. Sci Transl Med. 2012;4(136):136ra68.
9. Bettegowda C, Sausen M, Leary R, Kinde I, Wang Y, Agrawal N. Detection of circulating tumor DNA in early-and-late stage human malignancies. Sci Transl Med. 2014;6(224):224ra24.
10. Kinde I, Wu J, Papadopoulos N, Kinzler K, Vogelstein B. Detection and quantification of rare mtations with massively parallel sequencing. Proc Natl Acad Sci U S A. 2011;108(23):9530-9535.
11. Kennedy SR, Schmitt MW, Fo EJ, et al. Detecting ultralow-frequency mutations by Duplex Sequencing. Nat Protoc. 2014;9(11):2586-2606.
12. Thierry AR, Mouliere F, El Messaoudi S, et al. Clinical validation of the detection of KRAS and BRAF mutations from circulating tumor DNA. Nat Med. 2014;20(4):430-435.
13. Gevensleben H, Garcia-Murillas I, Graeser MK, et al. Noninvasice detection of HER2 amplification with plasma DNA digital PCR. Clin Cancer Res. 2013;19(12):3276-3284.
14. Abel GA. Does surveillance imaging after treatment for diffuse large B-cell lymphoma really work. J Clin Oncol. 2015;33(13):1427-1429.
15. Diaz L, Williams R, Wu J, et al. The molecular evolution of acquired resistance to targeted EGFR blockade in colorectal cancers. Nature. 2012;486(7404):537-540.
16. Amant F, Verheecke M, Wlodarska I, et al. Presymptomatic identification of cancers in pregnant women during noninvasive prenatal testing. JAMA Oncol. 2015;1(6):814-819.
17. Bianchi DW, Chudova D, Sehnert AJ, et al. Noninvasive prenatal testing and incidental detection of occult maternal malignancies. J Am Med Association. 2015;314(2):162-169.
18. Van Oers M, Tönissen E, Van Glabbeke M, et al. BCL-2/IgH polymerase chain reaction status at the end of induction treatment is not predictive for progression-free survival in relapsed/resistant folicular lymphoma: results of a prospective randomized EORTC 20981 phase III intergroup study. J Clin Oncol. 2010;28(13):2246 - 2252.
19. Vandenberghe P, Wlodarska I, Tousseyn T, et al. Non-invasive detection of genomic imbalances in Hodgkin/Reed-Sternberg cells in early and advanced stage Hodgkin's lymphoma by sequencing of circulating cell-free DNA: a technicalproof-of-principle study. Lancet Haematol. 2015;2(2):e55-65.
20. Steidl C, Diepstra A, Lee T, Chan F. Gene expression profiling of microdissected Hodgkin Reed-Sternberg cells correlates with treatment outcome in classical Hodgkin lymphoma. Blood. 2012;120(17):3530-3540.
21. Reichel J, Chadburn A. Flow-sorting and exome sequencing reveals the oncogenome of primary Hodgkin and Reed Sternberg cells. Blood. 2014;125(7):1061-1072.
22. Hohaus S, Giachela M, Massini G, et al. Cell-free circulating DNA in Hodgkin's and non-Hodgin's lymphomas. Ann Oncol. 2009;20(8):1408-1413.
23. Yu S, Lee S, Jiang P, et al. High-resolution profiling of fetal DNA clearance from maternal plasma by massive parallel sequencing. Clin Chem. 2013;59(8):1228-1237.
24. Liedtke M, Hamlin P, Moskowitz C, Zelenetz A. Surveillance imaging during remission identifies a group of patients with more favorable aggresive NHL at time of relapse: a retrospective analysis of a uniformly-treated patient population. Ann Oncol. 2006;17(6):909-913.
25. Roschewski M, Dunleavy K, Pittaluga S, Moorhead M, Pepin F. Circulating tumour DNA and CT monitoring in patients with untreated diffuse large B-cell lymphoma: a correlative biomarker study. Lancet Oncol. 2015;16(5):541-549.
26. Li M, Diehl F, Dressman D, Vogelstein B, Kinzler K. BEAMing up for detection and quantification of rare sequence variants. Nat Methods. 2006;3(2):95-97.
27. Glenn T. Field guide to next-generation DNA sequencers. Mol Ecol Resour. 2011;11(5):759-769.
28. Li J, Wang L, Mamon H, Kulke MH, Berbeco R, Makrogiorgos M. Replacing PCR with COLD-PCR enriches variant DNA sequences and redefines the sensitivity of genetic testing. Nat Med. 2008;14(5):579-584.
29. Makrigiorgos M. Novel methods for enrichment of mutations and differentially methylated sequences from liquid biopsy genomes. Molecular Diagnostics Europe, Circulating Cell-Free DNA. Lisbon, 2016.

Hematopoietic stem cells meet induced pluripotent stem cells technology

Maria Teresa Esposito*

School of Health, Sport and Bioscience, University of East London, Water Lane London E15 4LZ, UK.

E-mail: m.esposito@uel.ac.uk *doi:10.3324/haematol.2016.150755*

Hematopoietic stem cells (HSCs), the source of the entire blood cells repertoire, represent the first stem cells identified in an adult tissue, the bone marrow, and the first stem cells used as a therapy in humans, through bone marrow transplantation.[1] Although this therapeutic approach is well established and used to treat a variety of hematological conditions, including leukemia, several aspects limit its application. A lack of matched donors poses a serious barrier, especially for patients from ethnic minority backgrounds. Finding the perfect match between donor and recipient, and obtaining a large number of HSCs, represent two of the unmet major clinical challenges.

Development in a parallel area of stem cells research, induced pluripotent stem cells (iPS) technology, might provide new avenues to circumvent the limitations posed by the scarce number of HSCs available for transplantation. A decade ago the team led by Nobel prize winner Shinya